EXHIBIT 9

Harmony™
PRENATAL TEST

A simple blood test to detect common fetal trisomies in pregnancies of 10 weeks or more, based on directed analysis of DNA in maternal blood

Harmony™ comes to fetal trisomy testing

Accurate *Enhanced sensitivity and specificity*[1]

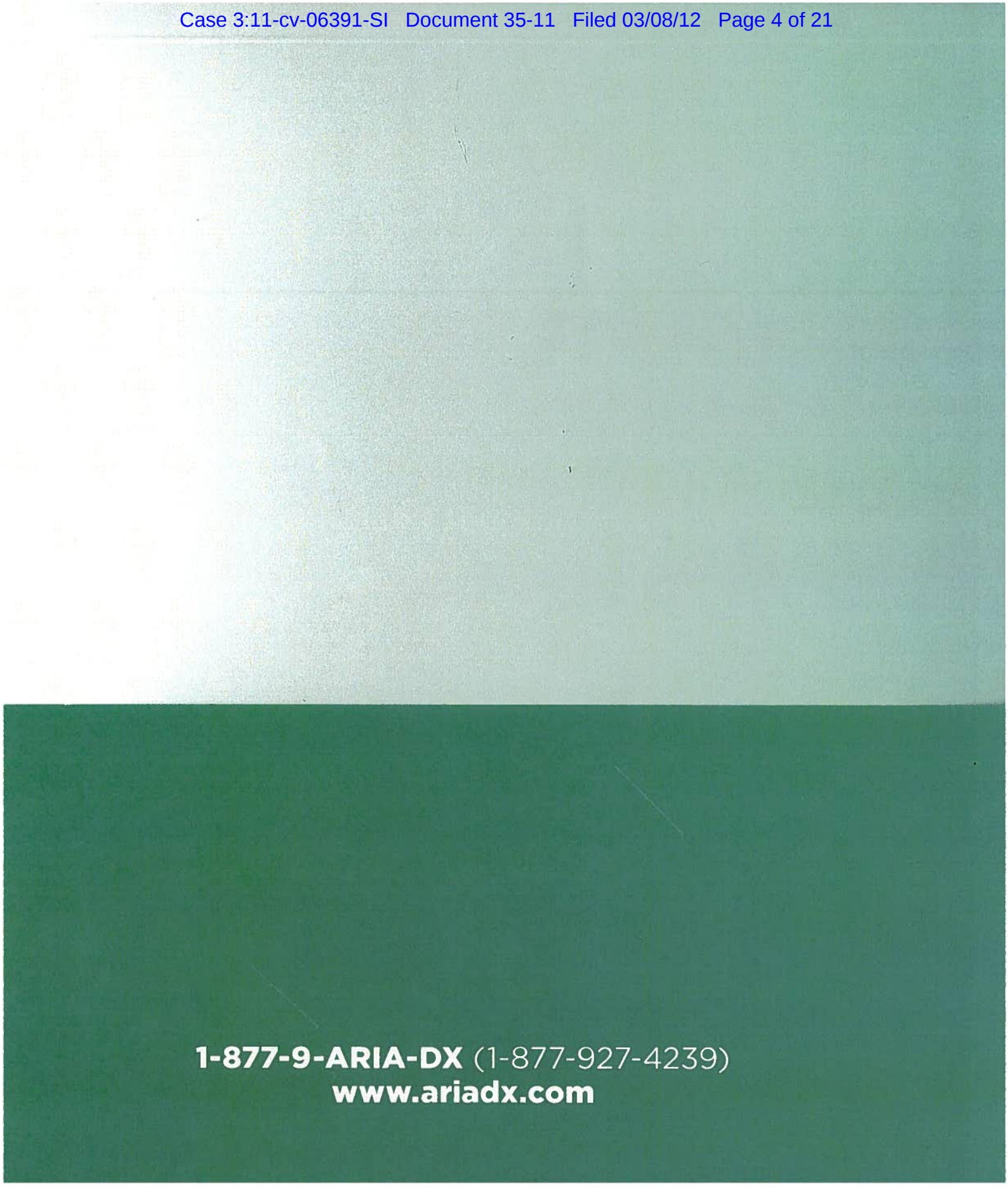
Safe *In-office maternal blood draw*

Affordable *Based on advanced DNA technology*[2]

Harmony™
PRENATAL TEST

About Aria Aria Diagnostics, Inc., is a molecular diagnostics company committed to providing safe, highly accurate, and affordable prenatal tests for maternal and fetal health. Led by an experienced team, Aria is using its proprietary technology to perform a directed analysis of cell-free DNA in blood. Aria's simple blood test equips pregnant women and their healthcare providers with reliable information to make decisions regarding their health, without creating unnecessary stress or anxiety.

REFERENCES **1.** Sparks AB, et al. *Prenat Diagn.* 2012 Jan 6:1-7. doi: 10.1002/pd.2922. **2.** Data on file.


1-877-9-ARIA-DX (1-877-927-4239)
www.ariadx.com

Aria™ DIAGNOSTICS

Harmony™ PRENATAL TEST

The newest advance in non-invasive fetal trisomy testing

Ask your provider

The following information is provided to inform you about the Harmony Prenatal Test clinical laboratory service. Talk to your healthcare provider before you decide if the Harmony Prenatal Test is appropriate for you.

Simple, safe, and accurate for you and your pregnancy

The Harmony Prenatal Test service is a non-invasive test that detects common fetal trisomies in pregnancies of 10 weeks or more, based on directed analysis of DNA in maternal blood.

What is a trisomy?

Humans have 23 pairs of chromosomes, which are strands of DNA and proteins that carry genetic information. A trisomy is a chromosomal condition that occurs when there are three copies of a particular chromosome instead of the expected two.

Trisomy 21 is due to an extra copy of chromosome 21 and is the most common trisomy at the time of birth. Trisomy 21 causes Down syndrome, which is associated with mild to moderate intellectual disabilities and may also lead to digestive issues and congenital heart defects.[1] It is estimated that Down syndrome is present in one out of every 740 newborns.[1]

Trisomy 18 is due to an extra copy of chromosome 18. Trisomy 18 causes Edwards syndrome and is associated with a high rate of miscarriage. Infants born with Edwards syndrome may have various medical conditions and a shortened lifespan. It is estimated that Edwards syndrome is present in approximately one out of every 5,000 newborns.[2]

Besides trisomy 21 and trisomy 18, there are other trisomies which are less common at birth or associated with milder medical conditions.

What will the Harmony Prenatal Test tell me and my provider?

The Harmony Prenatal Test service determines the risk of common fetal trisomies by measuring the relative amount of chromosomes in maternal blood.

How is the Harmony Prenatal Test different from other prenatal tests?

The Harmony Prenatal Test is based on the newest advances in non-invasive prenatal testing. It is a simple and safe blood test that has been shown in analytical studies to detect the risk of common fetal trisomies.[3]

Screening tests such as serum blood tests and ultrasound are also non-invasive, but have false-positive rates of up to 5% and miss detection of up to 30% of fetal trisomy 21 cases.[4] Such tests may falsely report a pregnancy as positive for a fetal trisomy when it is in fact negative (a false positive). Or they may falsely report a pregnancy as negative for a fetal trisomy when it is in fact positive (a false negative).

Diagnostic tests such as amniocentesis or chorionic villus sampling (CVS) are accurate for detecting fetal trisomies, but they are invasive and pose a risk for fetal loss.[5]

Who can get the Harmony Prenatal Test?

The Harmony Prenatal Test service can be ordered by healthcare professionals for women with pregnancies of at least 10 weeks' gestational age. The test is not for use in multiple pregnancies (such as twins) or egg-donor pregnancies.

Please let your healthcare team know if you have any additional questions about the Harmony Prenatal Test. For insurance and reimbursement questions, please call 1-877-9-ARIA-DX (1-877-927-4239).

The Harmony Prenatal Test has been developed and is performed as a laboratory test service by Aria Diagnostics, a CLIA-certified clinical laboratory.

1. U.S. National Library of Medicine. Genetics Home Reference. Down Syndrome. http://ghr.nlm.nih.gov/condition/down-syndrome. Accessed January 5, 2012.
2. U.S. National Library of Medicine. Genetics Home Reference. Trisomy 18. http://ghr.nlm.nih.gov/condition/trisomy-18. Accessed January 3, 2012.
3. Data on file, Aria Diagnostics. **4.** ACOG Practice Bulletin 77. Screening for fetal chromosomal abnormalities. *Obstet Gynecol.* 2007;109:217-227.
5. ACOG Practice Bulletin 88. Invasive prenatal testing for aneuploidy. *Obstet Gynecol.* 2007;110:1459-1467.

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Aria™ DIAGNOSTICS

Harmony™ PRENATAL TEST

El último avance en pruebas no invasivas de trisomía fetal

Consulte con su proveedor de atención médica

Se le proporciona la siguiente información para informarle acerca del servicio de laboratorio clínico de la Prueba prenatal Harmony. Consulte con su proveedor de atención médica antes de determinar si la Prueba prenatal Harmony es apropriado para usted.

Facil, segura y exacta para usted y su embarazo

El servicio de Prueba prenatal Harmony es una prueba no invasiva mediante la cual se detectan las trisomías fetales comunes en embarazos de 10 semanas o más, basándose en el análisis dirigido del ADN de la sangre materna.

¿Qué es una trisomía?

Los seres humanos tienen 23 pares de cromosomas, que son cadenas de ADN y proteínas que portan la información genética. Una trisomía es una anomalía del cromosoma que ocurre cuando hay tres copias de un cromosoma particular en lugar de las dos previstas.

La **trisomía 21** se debe a una copia extra del cromosoma 21 y es la trisomía más común en el momento del nacimiento. La trisomía 21 causa el síndrome de Down, el cual está asociado a discapacidades intelectuales de varios niveles, y puede también derivar en problemas digestivos y anomalías cardíacas congénitas.[1] Se estima que el síndrome de Down ocurre en uno de cada 740 recién nacidos.[1]

La **trisomía 18** se debe a una copia extra del cromosoma 18. La trisomía 18 causa el síndrome de Edwards, y está asociada a una alta tasa de abortos espontáneos. Los niños nacidos con el síndrome de Edwards pueden tener diversas afecciones médicas y una menor expectativa de vida. Se estima que el síndrome de Edwards está presente en aproximadamente uno de cada 5,000 recién nacidos.[2]

Además de las trisomías 21 y 18, existen otras trisomías menos comunes en el momento del nacimiento o que están asociadas a afecciones médicas más leves.

¿Qué nos dirá a mí y a mi proveedor de atención médicala la Prueba prenatal Harmony?

El servicio de Prueba prenatal Harmony determina el riesgo de trisomías fetales comunes mediante la medición de la cantidad relativa de cromosomas en la sangre materna.

¿En qué se diferencia la Prueba prenatal Harmony de otras pruebas prenatales?

La Prueba prenatal Harmony se basa en los últimos avances en pruebas prenatales no invasivas. Es un análisis de sangre facil y seguro, que en estudios analíticos ha demostrado detectar el riesgo de trisomías fetales comunes.[3]

Las pruebas de detección tales como las pruebas en suero sanguíneo y las ecografías tampoco son invasivas, pero tienen tasas de falsos positivos de hasta el 5% y fallan en la detección en hasta un 30% de casos de trisomías fetales 21.[4] Dichas pruebas pueden arrojar un resultado positivo incorrecto de trisomía fetal en un embarazo cuando, en realidad, el resultado es negativo (un falso positivo). O pueden dar un resultado negativo incorrecto de trisomía fetal en un embarazo cuando, en realidad, el resultado es positivo (un falso negativo).

Las pruebas diagnósticas tales como la amniocentesis o las pruebas en muestras de villuscoriónico (CVS, en inglés) son exactas para detectar trisomías fetales, pero son invasivas y representan un riesgo de pérdida del bebé.[5]

¿Quién puede hacerse la Prueba prenatal Harmony?

El servicio de Prueba prenatal Harmony puede ser solicitado por profesionales de atención médica para mujeres con embarazos de al menos 10 semanas de gestación. La prueba no es válida en embarazos múltiples (tales como gemelos) ni en embarazos concebidos mediante la donación de óvulos.

Hágale saber a su equipo de atención médica si tiene alguna pregunta adicional acerca de la Prueba prenatal Harmony. Para preguntas sobre seguro y reembolsos, llame al 1-877-9-ARIA-DX (1-877-927-4239).

La Prueba prenatal Harmony es un servicio de prueba de laboratorio desarrollado y ofrecido por Aria Diagnostics, un laboratorio clínico certificado por las Enmiendas para la Mejora de los Laboratorios Clínicos (ClinicalLaboratoryImprovementAmendments, CLIA).

1. U.S. National Library of Medicine. Genetics Home Reference. Down Syndrome. http://ghr.nlm.nih.gov/condition/down-syndrome. Acceso: 5 de enero de 2012.
2. U.S. National Library of Medicine. Genetics Home Reference. Trisomy 18. http://ghr.nlm.nih.gov/condition/trisomy-18. Acceso: 3 de enero de 2012.
3. Datos en archivos, Aria Diagnostics. **4.** ACOG Practice Bulletin 77. Screening for fetal chromosomal abnormalities. *Obstet Gynecol.* 2007;109:217-227.
5. ACOG Practice Bulletin 88. Invasive prenatal testing for aneuploidy. *Obstet Gynecol.* 2007;110:1459-1467.

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Aria DIAGNOSTICS

INSURANCE AND REIMBURSEMENT INFORMATION FOR PATIENTS

Harmony™ PRENATAL TEST

If you are considering the Harmony Prenatal Test, you may have questions regarding insurance coverage and reimbursement.

To help answer your questions, Aria Diagnostics offers the following services:

* Reimbursement Hotline
* Prompt Pay Program
* Financial Assistance Program
* Support for appeals with your insurance company when necessary

Aria™ DIAGNOSTICS

Will my insurance company cover my test? Who bills them?

Aria Diagnostics will bill your insurance company on your behalf and work directly with it on billing matters. Certain insurance companies will provide some payment for the test.

Whom can I call for help with billing or reimbursement questions?

Our Reimbursement Hotline specialists are available to assist you from 9AM to 7PM Eastern time, Monday through Friday, at **1-877-9-ARIA-DX (877-927-4239)**.
Please note that the billing office is closed on public holidays.

*The Prompt Pay Program is not available to patients with Medicare or Medicaid coverage. The Prompt Pay Program will not be offered in states where prohibited by law. Aria Diagnostics maintains the right to modify or discontinue the Prompt Pay Program or the Financial Assistance Program at any time.

Aria™ DIAGNOSTICS

Company Summary

Brief Description: Aria Diagnostics, Inc. is committed to providing safe, highly accurate and affordable prenatal tests for maternal and fetal health. Aria is currently developing a simple blood test to detect common fetal trisomies via directed DNA analysis. This test should equip pregnant women and their healthcare providers with reliable information to make decisions regarding their health, without creating unnecessary stress or anxiety.

Test Description: Harmony Prenatal Test™ clinical laboratory service for fetal trisomy detection. This test is being developed as a laboratory test service by Aria Diagnostics, a CLIA-certified clinical laboratory

Clinical Studies Overview:

Clinical Study	Description	Subject enrollment	Status
Proof of Concept	Initial demonstration of Aria's directed DNA analysis approach for T21 and T18 detection	298	Published
Blinded study (Aria)	Introduction of more accurate result reporting method; blinded study for T21 and T18 detection	338	Published
Blinded study (Nicolaides)	External validation of Aria's test method for T21 and T18 in first trimester pregnancies	400	Published
NICE Study (Non-Invasive Chromosomal Evaluation)	Multi-center (50 sites) validation study for T21 and T18	4,000 (estimate)	Enrolled
NITE (Non-Invasive Trisomy Evaluation)	Multicenter blinded study across European sites	500 (estimate)	Enrolling
NEXT study (Non-invasive EXamination of Trisomy)	Multicenter blinded study comparing Harmony Prenatal Test to combined first trimester screening	25,000 (estimate)	Enrolling

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Aria Diagnostics Leadership

John Stuelpnagel, DVM - Executive Chairman

John Stuelpnagel is Executive Chairman of Aria Diagnostics. Prior to Aria, John co-founded Illumina, Inc. and was instrumental in making Illumina one of the most successful life science companies. During his 12 years at Illumina, John served as the company's first CEO and most recently served as COO. John received his B.S. in Biochemistry and his Doctorate in Veterinary Medicine from UC Davis and his M.B.A. from UCLA.

Ken Song, MD - Chief Executive Officer

Ken Song brought together the initial team for Aria Diagnostics and serves as CEO. Ken is also responsible for leading clinical strategy and development. Ken is a board certified physician, has extensive clinical and basic science research experience, and was previously an investor at Venrock. Ken received his B.S. in Biology from the Massachusetts Institute of Technology and his M.D. from the UCSF.

Arnold Oliphant, PhD - Chief Scientific Officer

Arnold Oliphant serves as CSO of Aria Diagnostics and has led development of the company's proprietary assay platform. Arnold has over 20 years of industry experience in developing novel assays for research and clinical use at companies such as Myriad Genetics, Illumina, and Complete Genomics. Arnold received his B.A. in Genetics from the University of Utah and his Ph.D. in Genetics from Harvard University.

Todd Whitson - Vice President, Sales and Commercial Operations

Todd Whitson was the first commercial hire at Aria Diagnostics. He is responsible for building the commercial team with an emphasis on sales and operations. Prior to Aria, Todd led the integration of the combined oncology sales force for Genzyme Genetics and LabCorp. Todd has also led sales at Genzyme Genetics for reproductive diagnostic products. Todd received his BA in Business/Economics and Psychology from Lafayette College and a M.A. in Sports Administration from Richmond College.

Andrew Sparks, PhD - Vice President, Operations

Andrew Sparks is responsible for leading Aria's clinical lab operations, having successfully spearheaded development of Aria's assay platform. Andrew has over 15 years' experience developing and deploying large-scale genetic analysis technologies at companies such as Complete Genomics, Perlegen, and Affymetrix. Andrew received his B.S. in Biology from the University of Texas and his Ph.D. in Genetics from UNC.

Todd Rubano - Vice President, Informatics

Todd Rubano assumes responsibility at Aria Diagnostics for laboratory automation and information systems, business applications and other information technology. Prior to Aria, Todd led systems integration at Illumina, and he also led efforts at Myriad Genetics to automate the first commercially available DNA sequence-based test for breast cancer. Todd received his B.S. in Systems Engineering from University of Arizona and his M.E. in Engineering Systems from Colorado School of Mines.

Dianna DeVore, PhD, JD - Vice President, Intellectual Property and Legal Affairs

Dianna DeVore leads the IP and legal affairs of Aria Diagnostics. Dianna has over 16 years of legal experience, with a focus on intellectual property and technology transactions. She was a founder of the Silicon Valley-based law firm, Convergent Law Group. Dianna received her B.A in Biology and Art History from Johns Hopkins, her Ph.D. in Genetics from Yale University, and her J.D. from Stanford Law School.

Dan Puckett, MBA - Vice President, Finance & Administration

Dan Puckett is responsible for leading the finance and human resources functions at Aria Diagnostics. Dan came to Aria from Forest Laboratories where he served as Executive Director, Operations of Cerexa. Prior to Cerexa, Dan held senior finance and operations positions at Affymetrix, AOL, Viad, and BHP Billiton. Dan holds an M.B.A. from the University of San Francisco and a B.A. degree in accounting from Washington State University.

Aria™ DIAGNOSTICS

Abstract

Non-invasive Prenatal Detection and Selective Analysis of Cell-free DNA Obtained from Maternal Blood: Evaluation for Trisomy 21 and Trisomy 18

American Journal of Obstetrics and Gynecology, published online 25 January 2012,
http://www.sciencedirect.com/science/article/pii/S0002937812000610?v=s5

Andrew B. Sparks, Craig A. Struble, Eric T. Wang, Ken Song, Arnold Oliphant

Objective

To develop a novel biochemical assay and algorithm for the prenatal evaluation of risk for fetal trisomy 21 and 18 using cell-free DNA obtained from maternal blood.

Study Design

We assayed cfDNA from a training set and a blinded validation set of pregnant women, comprising 250 disomy, 72 trisomy 21 (T21), and 16 trisomy 18 (T18) pregnancies. We used Digital ANalysis of Selected Regions (DANSR) in combination with a novel algorithm, Fetal-fraction Optimized Risk of Trisomy Evaluation (FORTE) to determine trisomy risk for each subject.

Results

163/171 subjects in the training set passed quality control (QC) criteria. Using a Z statistic, 35/35 T21 cases and 7/7 T18 cases had Z statistic >3 and 120/121 disomic cases had Z statistic <3. FORTE produced an individualized trisomy risk score for each subject, and correctly discriminated all T21 and T18 cases from disomic cases. All 167 subjects in the blinded validation set passed QC and FORTE performance matched that observed in the training set correctly discriminating 36/36 T21 cases and 8/8 T18 cases from 123/123 disomic cases.

Conclusions

DANSR and FORTE enable accurate, scalable non-invasive fetal aneuploidy detection.

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New Data Publication in the *American Journal of Obstetrics & Gynecology* Highlights Proprietary Algorithm for Aria's Non-Invasive Prenatal Assay

Results Demonstrate Enhanced Accuracy in Detecting Both T21 and T18 Abnormalities

San Jose, Calif., Jan. 27, 2012 – Aria Diagnostics, a molecular diagnostics company, today announced the publication of a study showing that Aria's technology -- a directed non-invasive approach to cell-free DNA (cfDNA) analysis in maternal blood using a proprietary algorithm -- enabled non-invasive detection of Trisomy 21 (associated with Down syndrome) and Trisomy 18 (associated with Edwards syndrome). The data appear online in the *American Journal of Obstetrics & Gynecology* (AJOG) at http://www.sciencedirect.com/science/article/pii/S0002937812000610?v=s5.

"The encouraging results from this study suggest that the novel algorithm is a good complement to the directed cfDNA assay, as it allows for the calculation of an individualized risk score for Trisomy 18 and 21," explained Howard S. Cuckle, PhD, president of the International Society for Prenatal Diagnosis, professor at Columbia University Medical Center, New York. "The ability to selectively sequence the DNA, in combination with the algorithm, shows great promise for increasing efficiency and applicability with this technology."

The study examines use of Aria's highly multiplexed assay, known as Digital Analysis of Selected Regions (DANSR™), which utilizes innovative biochemistry to efficiently analyze patient samples. It also introduces an algorithm known as Fetal-fraction Optimized Risk of Trisomy Evaluation (FORTE™) that incorporates the results of the DANSR assay, the percentage of fetal DNA in the patient's sample and clinical information such as the maternal age to provide a risk score for each patient.

Titled, "Non-invasive Prenatal Detection and Selective Analysis of Cell-free DNA Obtained from Maternal Blood: Evaluation for Trisomy 21 and Trisomy 18," the study involved 338 women 18 years and older who were of at least 10 weeks gestational age and pregnant with only one fetus. They were divided into a training set designed to optimize the assay and algorithm, and a blinded test set used for validation. The algorithm produced individualized trisomy risk scores for T21 and T18 for each subject, and accurately classified all T21 and T18 cases from all non-trisomic cases.

"These data support previous research to show that our approach should make routine non-invasive testing for common trisomies possible and affordable," said John Stuelpnagel, DVM, executive chairman at Aria Diagnostics. "We are excited to see how the algorithm continues to perform with the Aria test in additional studies."

An independent blinded study evaluating the accuracy of Aria's test was also published online recently and is accessible at http://www.sciencedirect.com/science/article/pii/S0002937812000609?v=s5. This additional study, authored by Professor Kypros Nicolaides, MD, of Harris Birthright Research Centre for Fetal Medicine at King's College Hospital in the United Kingdom, was made possible by a grant from the Fetal Medicine Foundation.

About Aria Diagnostics (formerly Tandem Diagnostics)

Aria Diagnostics, Inc., is a molecular diagnostics company committed to providing safe, highly accurate and affordable prenatal tests for maternal and fetal health. Led by an experienced team, Aria is using its proprietary technology to perform a directed analysis of cell-free DNA in blood. Aria's simple blood test equips pregnant women and their healthcare providers with reliable information to make decisions regarding their health, without creating unnecessary stress or anxiety.

The company began operations in 2010 and is headquartered in San Jose, Calif. For more information, visit www.ariadx.com.

Aria™ DIAGNOSTICS

Abstract

Chromosome-selective sequencing of maternal plasma cell-free DNA for first-trimester detection of trisomy 21 and trisomy 18

Original Research Article

American Journal of Obstetrics and Gynecology, *published online 24 January 2012*,
http://www.sciencedirect.com/science/article/pii/S0002937812000609?v=s5

Ghalia Ashoor, Argyro Syngelaki, Marion Wagner, Cahit Birdir, Kypros H. Nicolaides

Objective
To assess the prenatal detection rate of trisomies 21 and 18 and false positive rate by chromosome-selective sequencing of maternal plasma cell-free DNA (cfDNA).

Study design
Nested case-control study of cfDNA was examined in plasma obtained before chorionic villous sampling from 300 euploid, 50 trisomy 21 and 50 trisomy 18 pregnancies at 11-13 weeks. Laboratory personnel were blinded to fetal karyotype.

Results
Risk scores for trisomy 21 and trisomy 18 were given for 397 of the 400 samples that were analyzed. In all 50 cases of trisomy 21 the risk score for trisomy 21 was ≥99% and the risk score for trisomy 18 was ≤0.01%. In all 50 cases of trisomy 18 the risk score for trisomy 21 was ≤0.01% and the risk score for trisomy 18 was ≥99% in 47 cases, 98.8% in one, 88.5% in one and 0.11% in one. In 3 (1%) of the 300 euploid pregnancies no risk score was provided because there was failed amplification and sequencing. In the remaining 297 the risk score for trisomy 21 was ≤0.01% and the risk score for trisomy 18 was ≤0.01% in 295 cases, 0.04% in one and 0.23% in one. Therefore, the sensitivity for detecting trisomy 21 was 100% (50/50), the sensitivity for trisomy 18 was 98% (49/50) and the specificity was 100% (297/297).

Conclusion
In this study, chromosome-selective sequencing of cfDNA separated all cases of trisomy 21 and 98% of trisomy 18 from euploid pregnancies.

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Aria Announces Publication of Independent Study in the *American Journal of Obstetrics & Gynecology* Using Aria's Technology

Study, Which Exclusively Examines Women in their First Trimester of Pregnancy, Provides Largest Data Set to Date for Trisomy 18 Pregnancies

San Jose, Calif., Jan. 26, 2012 – Aria Diagnostics, a molecular diagnostics company, today announced the publication of an independent blinded study that examines the accuracy of its non-invasive prenatal test for the detection of Trisomy 21 (associated with Down syndrome) and Trisomy 18 (associated with Edwards syndrome). The study showed that the test, coupled with a proprietary algorithm to assess individualized risk for these trisomies, distinguished 100 percent of T21 and 98 percent of T18 cases, while correctly identifying 100 percent of the non-trisomic samples. The data appear online in the *American Journal of Obstetrics & Gynecology* at http://www.sciencedirect.com/science/article/pii/S0002937812000609?v=s5.

"This constitutes a major breakthrough in the non-invasive detection of the two most common trisomies," said Professor Kypros Nicolaides, MD, of Harris Birthright Research Centre for Fetal Medicine at King's College Hospital in the United Kingdom and lead author of the paper. "I am now certain that this test will become a universal screening tool for Trisomies 21 and 18, provided the cost becomes comparable to that of current methods of sonographic and biochemical testing."

The study, titled, "Chromosome-Selective Sequencing of Maternal Plasma Cell-Free DNA for First-Trimester Detection of Trisomy 21 and Trisomy 18" was independently conducted and supported by a grant from the Fetal Medicine Foundation. The study assessed the ability of Aria's highly multiplexed assay, known as Digital Analysis of Selected Regions (DANSR™), to detect T21 and T18 by using chromosome-selective sequencing and to calculate risk scores with the proprietary Fetal-fraction Optimized Risk of Trisomy Evaluation (FORTE™) algorithm in 400 pregnant women at 11 to 13 weeks gestation. In addition to demonstrating the accuracy of Aria's technology, researchers reported that chromosome-selective sequencing requires 10 times less DNA sequencing than massively parallel shotgun sequencing (MPSS) approaches.

"This study provides firm support that Aria's technology has the potential to transform prenatal trisomy testing. Current prenatal testing involves a myriad of options that are confusing and either lack accuracy or pose harm to the pregnant woman and fetus," said Ken Song, chief executive officer of Aria Diagnostics. "We are developing a test that can greatly simplify the standard of care for pregnant women and give providers and patients confidence as a result of our highly accurate results."

About Aria Diagnostics (formerly Tandem Diagnostics)

Aria Diagnostics, Inc., is a molecular diagnostics company committed to providing safe, highly accurate and affordable prenatal tests for maternal and fetal health. Led by an experienced team, Aria is using its proprietary technology to perform a directed analysis of cell-free DNA in blood. Aria's simple blood test equips pregnant women and their healthcare providers with reliable information to make decisions regarding their health, without creating unnecessary stress or anxiety.

The company began operations in 2010 and is headquartered in San Jose, Calif. For more information, visit www.ariadx.com.

# # #

DOI: 10.1002/pd.2922 PRENATAL DIAGNOSIS

ORIGINAL ARTICLE

Selective analysis of cell-free DNA in maternal blood for evaluation of fetal trisomy

Andrew B. Sparks[1†], Eric T. Wang[1†], Craig A. Struble[1], Wade Barrett[1], Renee Stokowski[1], Celeste McBride[1], Jacob Zahn[1], Kevin Lee[1], Naiping Shen[1], Jigna Doshi[1], Michel Sun[1], Jill Garrison[1], Jay Sandler[1], Desiree Hollemon[1], Patrick Pattee[1], Aoy Tomita-Mitchell[2], Michael Mitchell[2], John Stuelpnagel[1], Ken Song[1]* and Arnold Oliphant[1]

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*Correspondence to: Ken Song. E-mail: ksong@ariadx.com
†Authors contributed equally to the work.

ABSTRACT

Objective To develop a novel prenatal assay based on selective analysis of cell-free DNA in maternal blood for evaluation of fetal Trisomy 21 (T21) and Trisomy 18 (T18).

Methods Two hundred ninety-eight pregnancies, including 39 T21 and seven T18 confirmed fetal aneuploidies, were analyzed using a novel, highly multiplexed assay, termed digital analysis of selected regions (DANSR™). Cell-free DNA from maternal blood samples was analyzed using DANSR assays for loci on chromosomes 21 and 18. Products from 96 separate patients were pooled and sequenced together. A standard *Z*-test of chromosomal proportions was used to distinguish aneuploid samples from average-risk pregnancy samples. DANSR aneuploidy discrimination was evaluated at various sequence depths.

Results At the lowest sequencing depth, corresponding to 204 000 sequencing counts per sample, average-risk cases where distinguished from T21 and T18 cases, with *Z* statistics for all cases exceeding 3.6. Increasing the sequencing depth to 410 000 counts per sample substantially improved separation of aneuploid and average-risk cases. A further increase to 620 000 counts per sample resulted in only marginal improvement. This depth of sequencing represents less than 5% of that required by massively parallel shotgun sequencing approaches.

Conclusion Digital analysis of selected regions enables highly accurate, cost efficient, and scalable noninvasive fetal aneuploidy assessment. © 2012 John Wiley & Sons, Ltd.

Funding sources: None
Conflicts of interest: None declared

INTRODUCTION

Prenatal testing for fetal chromosomal aneuploidies is commonly practiced and endorsed by professional medical organizations (ACOG Practice Bulletin No. 77).[1,2] Prenatal testing currently encompasses both screening and diagnostic modalities. Screening involves analysis of serum markers and/or ultrasound interpretation of fetal measurements such as nuchal translucency, but has suboptimal sensitivity and specificity (ACOG Practice Bulletin No. 77). [1] Diagnostic testing includes invasive procedures such as chorionic villus sampling (CVS) or amniocentesis, and although these tests are highly accurate, they come at significant health risks to the fetus and mother, including the potential loss of a healthy fetus.[3]

For the past several decades, numerous efforts have been pursued to develop a maternal blood test with improved accuracy for the detection of major fetal aneuploidies. Such blood tests could improve current screening practices for fetal aneuploidy. Initial efforts targeting isolation and analysis of circulating fetal cells in the maternal bloodstream have not proven successful, because of the challenges in detecting sufficient fetal cell numbers in circulation.[4–6] By contrast, analysis of cell-free DNA (cfDNA) in maternal circulation has shown promise for evaluation of fetal aneuploidy. Several groups have demonstrated the use of massively parallel DNA shotgun sequencing (MPSS) to assay for fetal Trisomy 21 (T21) from cfDNA in maternal blood.[7–10] More recently, several pilot studies have shown the ability of MPSS to assay for Trisomy 18 (T18) and Trisomy 13 (T13) from cfDNA in maternal blood, although with more variable results.[10,11]

Whereas MPSS has demonstrated robust technical performance, its cost and complexity, including the complexity

of data analysis, creates challenges for broad clinical adoption. Because MPSS analyzes random genomic fragments from all chromosomes, large amounts of unutilized sequencing data are generated. For example, in one study involving pregnant women with T21 fetuses, a mean of 10 800 000 sequencing reads per sample were generated, but on average, only 32 000 (0.3%) chromosome 21 sequences were utilized for aneuploidy detection.[7] Efforts to enrich for cfDNA from chromosomes of interest prior to MPSS have been described, but to date these approaches have not been used for detection of any genetic conditions.[12]

To address these limitations, we have developed a method called digital analysis of selected regions (DANSR™), which selectively evaluates specific genomic fragments from cfDNA. By enabling selective analysis of cfDNA, DANSR provides for more efficient use of sequencing to evaluate fetal aneuploidy. We report our initial investigation of this novel and promising method in a study of 298 pregnant women.

METHODS

Study population

A cohort comprised of women with singleton pregnancies was prospectively enrolled at selected prenatal care centers in the United States. Institutional Review Board approval was obtained for the study at all participating centers, and appropriate informed consent was obtained for all study participants. Average-risk women that had not undergone any invasive testing at the time of blood collection were enrolled and constituted the average-risk cases. The confirmed aneuploid cases consisted of pregnant women with T21 and T18 pregnancies confirmed via invasive testing with confirmatory fluorescent *in situ* hybridization and/or karyotype analysis.

Sample preparation

An average of 8 mL of blood was collected from each subject into a Cell-free™ DNA tube (Streck) or ethylenediaminetetraacetic acid (EDTA) tube. An average of 5 mL of plasma was isolated from each sample via a double centrifugation protocol of 1600 $\times g$ for 10 min, followed by 16 000 $\times g$ for 10 min, after a tube transfer following the first spin. cfDNA was isolated from plasma using the Dynabeads® Viral NA DNA purification kit (Dynal) protocol, with minor modifications. An average of 17 ng cfDNA was isolated from each patient sample and arrayed into individual wells of a 96-well microtiter plate.

Digital analysis of selected regions assay

Digital analysis of selected regions enables simultaneous quantification of hundreds of loci by cfDNA-dependent catenation of two locus-specific oligonucleotides via an intervening 'bridge' oligo to form a PCR template. We designed DANSR assays corresponding to 384 loci on each of chromosomes 18 and 21 (Figure 1). We selected loci to have sequences unique to the chromosomes of interest, to have uniform locus-specific oligo melting temperatures, to have minimal complementarity with universal amplification sequences, and to not coincide with known polymorphisms and copy number variants. No explicit selection for chromosome location was employed; Figure 2 depicts the distribution of the selected loci along each chromosome.

Three oligonucleotides (IDT) per locus were used: a left oligo consisting of a 5′ universal amplification tail (TACACCGGCGT-TATGCGTCGAGAC) followed by a locus-specific left sequence, a 5′-phosphorylated middle oligo, and a right oligo consisting of a 5′-phosphorylated locus-specific right sequence followed by a 3′ universal amplification tail (ATTGCGGGGACCGAT-GATCGCGTC). Oligonucleotides were pooled together to create a single DANSR assay oligo pool.

Cell-free DNA in each well of the microtiter plate was end labeled by incubation at 37°C for 1 h in a 30 µL reaction containing 150 pmol biotin-16-dUTP (Roche), 12U TdT (Enzymatics) and 1X TdT buffer (Enzymatics). After isopropanol precipitation to remove free biotin, cfDNA was resuspended in 10 mM Tris-HCl pH8.0, 0.1 mM EDTA (TE) and immobilized onto 100 µg myOneC1™ magnetic beads (Dynal) in a 50 µL hybridization reaction consisting of 60 mM

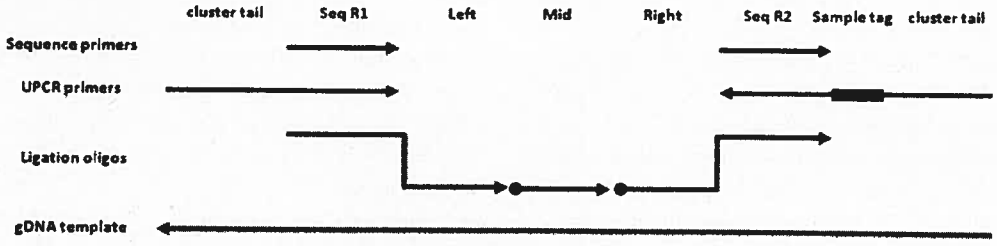

Figure 1 Schematic of DANSR assay. Arrows and dots indicate 3'OH and 5'PO_4 moieties, respectively. When the left, middle, and right ligation oligos hybridize to their cognate genomic DNA (gDNA) sequences, their termini form two nicks. Ligation of these nicks results in the creation of an amplifiable template using the indicated UPCR primers. UPCR with 96 distinct right UPCR primers enables pooling and simultaneous sequencing of 96 different UPCR products on a single lane. The UPCR primers also contain left (TAATGATACGGC-GACCACCGA) and right (ATCTCGTATGCCGTCTTCTGCTTGA) cluster tail sequences that support cluster amplification. Sequencing of the locus specific 56 bases and the seven sample specific bases are accomplished using read one (GATCTACACCGGCGTTATGCGTCGAGAC) and read two primers (TCAAGCAGAAGACGGCATACGAGAT) respectively

Figure 2 Chromosomal locations of selected loci. The selected loci are plotted as vertical lines against the Genome Reference Consortium human build 37 chromosome 21 in panel A and chromosome 18 in panel B. The plots' physical spans are indicated with boxes on their respective karyograms

Tris-HCl pH8.0, 6 mM EDTA, 300 mM $NaCl_2$, 35% formamide, 0.1% Tween-80, and 4 nM each locus specific DANSR oligonucleotides. The mixture was heated to 70 °C and annealed to 25 °C over 2 h, after which the beads were magnetically immobilized to the side of the microtiter plate well. After washing with 50 µL wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM $NaCl_2$), the beads were resuspended in a 50 µL reaction containing 1 U Taq ligase (Enzymatics) and 1X Taq ligase buffer (Enzymatics) and incubated at 37 °C for 1 h. The beads were washed twice with wash buffer and resuspended in 30 µL TE. The ligation product was eluted from the beads by incubation at 95 °C for 3 min and amplified by PCR for 26 cycles in a 50 µL reaction containing 1U Pfusion polymerase (Fermentas), 1X Pfusion buffer, 200 µM dNTPs (Enzymatics), 200 nM left universal PCR (UPCR) primer (TAATGATACGGCGACCACCGAGATCTACAC-CGGCGTTATGCGTCGAGAC), and 200 nM right UPCR primer (TCAAGCAGAAGACGGCATACGAGATNNNNNNNAAACGACG-CGATCATCGGTCCCCGCAAT), where NNNNNNN represents a 7 base sample index enabling 96 sample multiplexed sequencing. UPCR products from a single 96-well microtiter plate were pooled in equal volume, and the pool of UPCR products (library) was purified with AMPure XP (Beckman), according to the manufacturer's instructions. Each purified library was used as a template for cluster amplification on a TruSeq™ v2 SR cluster-kit flow-cell (Illumina), according to the manufacturer's protocols. The slide was processed on an Illumina HiSeq 2000™ to produce a 56 base locus-specific sequence and a 7 base sample tag sequence from each cluster.

Data analysis

Sequencing reads with fewer than three mismatches against the expected sequence for each selected genomic location were counted. The median percentage of sequence reads that mapped to selected loci was 96%. Sequence counts within a sample were highly uniform across loci; on average, the sequence counts within a sample of 90% of loci fell within a 2.14-fold range. Sequence counts were normalized by systematically removing sample and genomic location biases via median polish.[13,14] Specifically, the median count per locus within each sample was scaled to 1000. The counts were then log transformed to make chromosome, locus, and sample biases additive. The log transformed data were summarized using the linear model,

$$Y_{ijk} = \mu_k + \alpha_{ik} + \beta_{jk} + \varepsilon_{ijk},$$

where Y_{ijk} is the log count for locus i, sample j and chromosome k, μ_k is the bias associated with chromosome k, α_{ik} is the bias associated with location i on chromosome k, β_{jk} is the bias associated with sample j at chromosome k, and ε_{ijk} represents the error residuals in the model. The parameters of this model were estimated using the robust median polish technique.[13]

Once bias terms were estimated, normalization consisted of modifying terms to remove the associated biases. Systematic biases associated with chromosomes and loci were considered irrelevant for DANSR and were normalized by setting μ_k to an arbitrary value ($\log_2(1000)$) and $\alpha_{ik}=0$, respectively. The sample by chromosome interaction β_{jk} is essential for capturing aneuploid status and was therefore retained in the model. The residuals ε_{ijk} were also maintained. After modifying bias terms, the adjusted Y_{ijk} values were antilogged to generate normalized counts for the downstream analysis.

Normalized sequence counts from assays for 384 genomic regions on chromosomes 18 and 21 were used to calculate a standard Z-test of proportions

$$Z_j = \frac{p_j - p_0}{\sqrt{\frac{p_0(1-p_0)}{n_j}}}$$

where p_j is the observed proportion of representation for a given chromosome of interest in a given sample j, p_0 is the median expected proportion for the given test chromosome, and n_j is the sum of the mean count for each chromosome. p_j was obtained by taking the 20% trimmed mean count across all chromosome k loci for sample j and dividing by the 20% trimmed mean count across all loci (both chromosomes 21 and 18) for sample j, and p_0 was defined as the median of all p_j's within a sequencing lane. Because p_j and p_0 were computed after the normalization, no additional adjustments

or corrections were made. *Z*-statistic standardization was performed via an iterative censoring approach. At each iteration, the samples outside of three median absolute deviations were removed. At the end of ten iterations, the mean and standard deviation were calculated using only the uncensored samples. All samples were then standardized against this mean and standard deviation. The Kolmogorov–Smirnov test,[15] and Shapiro–Wilk's test [16] were used to test for the normality of the average-risk samples' *Z* statistics.

The separation distance was calculated by taking the difference (in *Z* statistic) between the lower 5th percentile of the affected and the upper 95th percentile of the average-risk samples.

RESULTS

A total of 298 plasma samples were analyzed in this study, including 39 T21 samples, seven T18 samples, and 252 samples from average-risk pregnant women. Table 1 summarizes demographic characteristics of the subjects included in the study. The median maternal age was 31 years (range 18–44 years) for all subjects. The median maternal ages for average-risk, T21, and T18 pregnancy subjects were 30 years (range 18–41), 36 years (range 18–43), and 36 years (range 29–44), respectively. The overall median gestational age was 13.4 weeks. The median gestational ages for the average-risk, T21, and T18 pregnancy subjects were 12.9 weeks, 20.0 weeks, and 17.3 weeks, respectively. The higher median maternal and gestational ages for subjects with confirmed aneuploidy were expected, given that a portion of these women had already undergone invasive testing and represented a higher-risk population.

Figure 3 shows the distribution of *Z* statistics for average-risk and T21 plasma samples at different sequencing depths. Figure 4 shows the distribution of *Z* statistics for average-risk and T18 plasma samples at different sequencing depths. In both sets of samples, the average-risk samples have a mean *Z* statistic close to zero, whereas the aneuploid samples have

Table 1 Demographic characteristics of the 298 pregnant women

Clinical parameter	Classification	Total	Median	Mean	Range
Maternal age (years)	All	298	31	30.4	[18,44]
	Avg risk	252	30	29.6	[18,41]
	T21	39	36	34.2	[18,43]
	T18	7	36	37.3	[29,44]
Gestational age (weeks)	All	298	13.4	15.6	[7,35.4]
	Avg risk	252	12.9	14.8	[7,33.1]
	T21	39	20.0	20.5	[13.4,35.4]
	T18	7	17.3	17.9	[11.1,23.6]

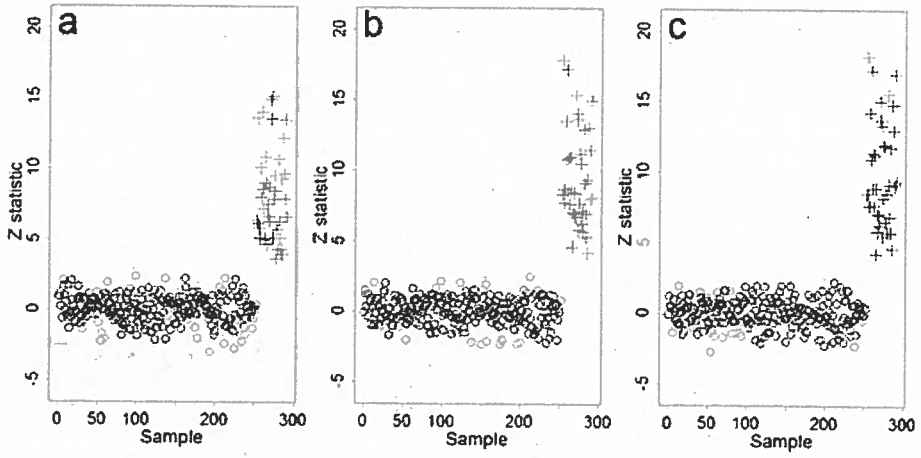

Figure 3 T21 *Z* statistics at various median per-sample counts. At each median per-sample count, the average-risk samples (open circles) were normally distributed around zero, while the T21 samples (crosses) were at least three standard deviations away. The separation distance was calculated by taking the difference (in *Z* statistic) between the 5th percentile of the affected samples and the 95th percentile of the average-risk samples. When the median per-sample count was 204 000 (a), the separation distance between average-risk and T21 samples was 4.2. When the median per-sample count was increased to 410 000 (b) and 620 000 (c), the resulting separation distances increased to 5.2 and 5.4, respectively

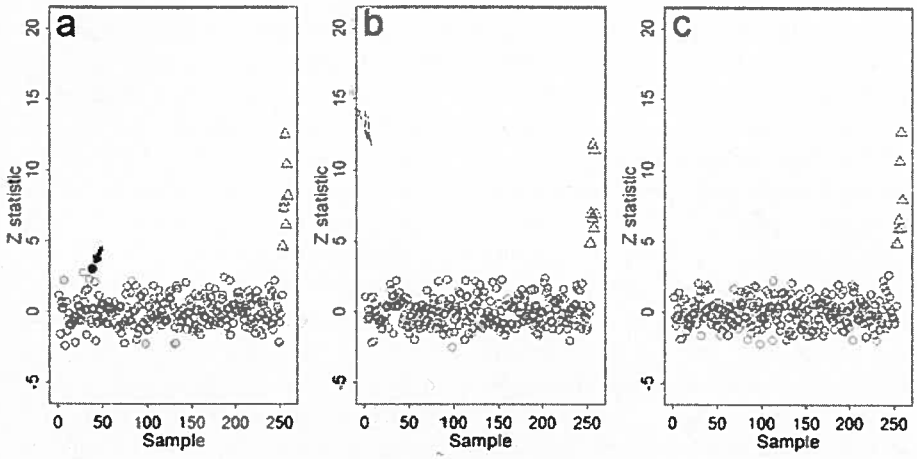

Figure 4 T18 *Z* statistics at various median per-sample counts. At a median per-sample count of 204 000 (a), the separation distance between average-risk samples (open circles) and T18 samples (triangles) was 4.9. When the median per-sample count was increased to 410 000 (b) and 620 000(c), the separation distances were both 5.2. When the median per-sample count was 204 000, one average-risk sample received a *Z* statistic of 3.005 (a; black dot, arrow). This sample subsequently received a *Z* statistic of less than three when we increased the median per-sample count to 410 000

Table 2 Summary statistics of the samples at different sequencing depths. The median per-sample count is the median of total counts across all samples. The percentage of sequences mapped was calculated by dividing the number of sequences mapped by the total number of observed sequences. The separation distance was calculated by taking the difference (in *Z* statistic) between lower 5th percentile of the affected samples and the upper 95th percentile of the average-risk samples

Median per-sample count (×1000)	Median % mapped	T21 separation (SD)	T18 separation (SD)	Avg risk *Z* statistics SD
204	96.6	4.2	4.9	1.02
410	96.8	5.2	5.2	0.99
620	96.8	5.4	5.2	1.00

Z statistics greater than 3.6. The *Z* statistics for the average-risk samples is normally distributed; Kolmogorov and Shapiro–Wilk's tests of normality showed $p < 0.50$ and $p < 0.13$, respectively. The standard deviation of the *Z* statistic for average-risk samples was approximately one (range 0.99–1.02), as shown in Table 2. Figures 3 and 4 demonstrate that the separation between the average-risk and aneuploid samples increases as the median sequencing counts per sample increases. Using a *Z* statistic cut-off of three to distinguish between average-risk and aneuploid samples, all aneuploid samples were correctly classified. At the lowest sequencing depth, one of 252 average-risk samples (0.4%) had a *Z* statistic of 3.005 when evaluating for T18 (Figure 2(a)). At higher sequencing depths, this sample had a *Z* statistic < 3.

To quantify the distinction between euploid and aneuploid samples, we calculated the separation distance as the difference between the *Z* statistic value of the upper 95th percentile of the average-risk samples and the lower 5th percentile of the affected samples. The separation distance for T21 and T18 samples increases with sequencing depth (Table 2). The greatest benefit in separation distance for both T21 and T18 was realized when increasing the median sequencing counts per sample from 204 000 to 410 000. Increasing the sequencing counts per sample from 410 000 to 620 000 had marginal benefit for T21 and no statistical benefit for T18. The improved separation resulting from increasing sequence depth beyond 204 000 counts per sample is consistent with the finding that at the lowest depth of sequencing, one average-risk sample had a *Z* statistic slightly greater than three (3.005), whereas with deeper sequencing, the *Z* statistic of all average-risk samples was less than three.

DISCUSSION

We have demonstrated that DANSR enables efficient and selective evaluation of cfDNA from maternal blood for fetal aneuploidy. We analyzed 298 plasma samples from pregnant women, including 39 T21 and seven T18 cases. Previous studies with MPSS have used a *Z* statistic cut-off of three standard deviations to classify cases as aneuploid or euploid.[7,9] Using a similar statistical analysis, we correctly distinguished all aneuploid cases from average-risk cases using as few as 420 000 reads per sample.

Digital analysis of selected regions has several advantages compared with MPSS as an assay for aneuploidy. First, the

fraction of raw sequencing reads that map to expected loci exceeds 96% with DANSR; by contrast, studies using MPSS report mapping rates of 20% to 50%.[7,8] Second, DANSR produces sequence data only from chromosomes of clinical interest; by contrast, MPSS produces data from all chromosomes, irrespective of their relevance to analysis of aneuploidy. Taken together, the DANSR advantages of mapping efficiency and selective analysis of specific chromosomes result in a greater than tenfold improvement on the sequencing cost and throughput of MPSS. DANSR thus has the potential to significantly lower the cost of noninvasive prenatal testing, which may make it economically practical for broad clinical use.

The sequencing cost and throughput advantages conferred by DANSR do not come at the cost of additional process complexity; DANSR library preparation requires a similar amount of time and effort as MPSS library preparation. The DANSR assay entails five major steps: biotinylation, precipitation, hybridization, ligation, and PCR. By comparison, Illumina's library preparation protocol entails six steps: end repair, AMPure XP, adenylation, adaptor ligation, AMPure XP, and PCR.

Because MPSS uses DNA from the entire genome, it theoretically requires less blood/plasma/cfDNA per patient than DANSR. In this study, we analyzed samples with a range of 5 to 11 mL of blood, yielding a range of 2.8 to 6.5 mL plasma, and a range of 5 to 60 ng cfDNA. We observed no difference in performance between samples with small versus large quantities of blood, plasma, or cfDNA. Although this suggests a lower amount of input material could be used, a 10 mL blood draw is clinically reasonable and standard. One potential advantage of MPSS over selective analysis is that MPSS data is collected from all chromosomes, allowing for the theoretical possibility of identification of aneuploidy events involving any chromosome. In this study, we focused our analysis on chromosomes 18 and 21, and found that 384 loci per chromosome was sufficient to enable aneuploidy discrimination. We recently evaluated cfDNAs using a single DANSR pool consisting of assays for more than 5000 loci (data not shown), and observed no substantial adverse effects of higher assay multiplex on data quality. This suggests that DANSR could be extended to include other chromosomes of clinical relevance.

In this study, we examined the effect of depth of sequencing on the ability of DANSR to discriminate between average-risk and aneuploid cases. We demonstrated that increasing the depth of sequencing results in better separation between average-risk and aneuploid cases. The benefit of increased sequencing depth likely reflects sampling noise reduction at the individual locus level. The incremental benefit of increasing sequencing coverage needs to be weighed against the additional sequencing costs. However, since these data were generated, several sequencing technology companies have made recent strides toward higher throughput. For example, a single sequencing lane on the Illumina HiSeq 2000 using version three chemistry can produce 150 million high quality counts, which readily accommodates 96 samples even at the highest sequencing depth explored in this study.

Although this study demonstrates the promise of selective cfDNA analysis, there are several caveats. First, the putative nontrisomy cases in this study were comprised of average-risk women whose fetal ploidy status was not independently confirmed. However, because the average age of the normal cases was 30 years, the likelihood of a T21 or T18 fetus being present in this cohort was very low. Second, the confirmed aneuploid cases were of older gestational age. It has been reported that the amount of fetal DNA in maternal blood increases with gestational age, although the effect is most pronounced in the third trimester.[17] Higher fetal DNA amounts may bias the results for the confirmed aneuploid cases, but the likelihood of this is low, because the majority of cases were in the second trimester or earlier. Future studies in which all the cases are confirmed (either by genotype or phenotype) to be diploid or triploid, and in which cases are matched for gestational age, are warranted. Lastly, this study was intentionally not blinded, because it represented our initial exploration of selective analysis of cfDNA for fetal aneuploidy using the DANSR assay. We are currently conducting prospective blinded studies to further validate the DANSR approach.

CONCLUSION

Digital analysis of selected regions represents a novel and promising approach to prenatal testing. By enabling selective analysis of cfDNA for evaluation of fetal aneuploidy, DANSR has the potential to significantly reduce test costs compared with MPSS approaches. In addition, DANSR may enable the study of other genetic conditions, including subchromosomal copy number variations, which are not as amenable to analysis by techniques such as MPSS. Prioritization of which genetic conditions to evaluate will require consideration of medical necessity, and the practicality of obtaining sufficient clinical samples for validation.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Non-invasive detection of fetal trisomy is feasible using massively parallel shotgun sequencing, which evaluates cfDNA fragments without regard to their chromosome of origin.

WHAT DOES THIS STUDY ADD?

- This study demonstrates for the first time non-invasive detection of trisomy 21 and trisomy 18 using selective sequencing of cfDNA from specific chromosomes, a dramatically more efficient and scalable method.

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